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PATENT
ATTORNEY DOCKET NO. 04148-00033

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Joost Dick de Bruijn, Sandra Claudia da Silva Madureira Mendes, and Clemens Antoni van Blitterswijk	Examiner:
Serial No.: 10/730,770	Art Unit:
Filed: December 8, 2003	
Title: ASSAY FOR PREDICTING CELL ACTIVITY	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

TRANSMITTAL LETTER

In regard to the above identified application, we are transmitting herewith the attached:

1. Transmittal of Priority Document,
2. Certified Copy of EP 01202220.8, and
3. Return postcard.

No additional fee is required. The Commissioner is hereby authorized to charge any additional fees or credit overpayment to Deposit Account No. 19-0733.

Respectfully submitted,

Dated: February 11, 2004

By ______________________

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USSN 10/730,770
Express Mail Receipt No. EV396913518 US

EV396913518US

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TRANSMITTAL OF PRIORITY DOCUMENT

Applicants submit herewith a certified copy of European Patent Application No. 01202220.8, from which the above-referenced U.S. patent application claims priority. No fee is due. Please apply any other charges or any credits to Deposit Account No. 19-0733.

Respectfully submitted,

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Europäisches Patentamt | European Patent Office | Office européen des brevets

Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

01202220.8

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

I.L.C. HATTEN-HECKMAN

Anmeldung Nr:
Application no.: 01202220.8
Demande no:

Anmeldetag:
Date of filing: 08.06.01
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

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PAYS-BAS

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Assay for predicting cell activity

In Anspruch genommene Prioriät(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

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G01N33/48

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR

EPO - DG 1

08. 06. 2001

(75)

Title: Assay for predicting cell activity

The invention relates to an *in vitro* method for determining the capacity of cells to induce bone formation *in vivo*. The invention further relates to a kit comprising the means to carry out said method.

The increasing demand for organ and tissue transplants has motivated several universities and private companies to develop research in the field of tissue engineering. At present, and with regard to the repair of skeletal defects, numerous investigators have purposed the use of autologous cultured tissue approaches as an alternative to the traditional bone grafting therapies. The engineering of bone tissue is based on the idea of seeding a suitable implant material with a patient's own cells that, during *in vitro* culture and prior to transplantation into the defect site, will form a bone tissue coating over the material surface.

The bone marrow stromal cell population is known to contain progenitors capable of differentiation into the mesenchymal lineages of bone, cartilage, fat and other connective tissues. Therefore, they constitute an interesting population for use in cell therapies. Furthermore, bone marrow stromal cells can be easily isolated from the patients marrow, extensively expanded during *in vitro* culture and, finally, induced to further differentiate into the relevant lineage. The *in vitro* and *in vivo* osteogenic potential of adult human bone marrow stromal cells (HBMSC) cultured on a porous calcium phosphate material has already been reported.

However, in several of these studies, *in vivo* bone induction by HBMSC cultures did not occur in all of the tested patients. Moreover, osteoinductivity of the cultures was found to decrease with patient age. Therefore, the development of an analysis method that will allow predicting *in vitro*, and in the early stages of proliferation, the performance of the tissue-engineered implants in an *in vivo* situation is of extreme importance.

The international patent application 94/26872 describes a method for assessing bone cell activity, wherein bone cells are cultured *in vitro* on a

thin calcium phosphate film. However, this method merely provides information on the resorptive activity of osteoclasts. It is not aimed at resulting in a determination of the capacity of bone cells taken in a biopsy to induce *de novo* bone formation *in vivo* after implantation in the form of a tissue engineered bone construct. Moreover, the method involves a lengthy and cumbersome procedure.

The present invention aims to provide a method by which the capacity of bone cells to induce bone formation *in vivo* can be determined *in vitro*. It is desired that the objective method is sufficiently simple to perform and has a sufficient level of sensitivity.

Surprisingly, it has been found that a relationship exists between the effect of an osteogenic differentiation factor, such as dexamethasone (dex), on the expression of alkaline phosphatase (ALP) by a bone cell population and the capacity of the bone cell population to induce bone formation *in vivo*. Accordingly, the desired determination of osteogenic potential can be achieved by comparing the expression of ALP by bone cells cultured in the presence of dex with said expression by bone cells cultured without dex.

Thus, the invention specifically relates to a method for determining *in vitro* the capacity of a cell population to induce bone formation *in vivo* comprising the steps of:

a) providing a sample of a cell population;

b) dividing said sample into a first and a second part containing an equal number of cells;

c) culturing the first part in the presence of an osteogenic differentiation factor;

d) culturing the second part in the absence of an osteogenic differentiation factor;

e) labelling the cells of the first and second parts with an antibody specific for alkaline phosphatase;

f) quantitatively determining degrees of expression of alkaline phosphatase expressed by the cells in each part; and

g) comparing the degrees of expression of alkaline phosphatase of the first part and the second part thereby providing a measure for the capacity of the bone cell population to induce bone formation *in vivo*.

A method according to the invention allows detection of cultures with low osteoinductivity, indicating the need for a second biopsy procedure or making possible further enhancement of the bone forming capacity of the cultures through the use of bone growth factors, such as bone morphogenetic proteins, that are known to posses a strong stimulatory effect on osteogenic commitment and differentiation of HBMSC. Furthermore, the present method can be used as a quality control step prior to transplantation of the implant into a patient defect site. A method according to the invention, therefore, reduces the frequency of cases in which bone induction *in vivo* is not achieved, thereby preventing failures of surgical procedures for bone implantations or substitutions.

The cell population of which the osteogenic potential can be determined in a method according to the invention can in principle be any sample of cells that are at least to some extent capable of undergoing differentiation to bone cells, such as osteoblasts and/or osteoclasts. Preferably, the bone cell population comprises human bone marrow stromal cells and/or human osteoprogenitor cells.

It is preferred that the sample of the cell population is obtained through a biopsy from a patient who has to undergo surgery for implantation of a bone prostheses or construct. The biopsy may be taken by any conventional means. Suitable locations for taking the biopsy are the iliac crest, the spine, the mandibula, and the acetobular fossa.

To perform a method according to the invention, the sample is first divided into two equal parts. This means that both parts should contain

substantially the same number of cells, which can be achieved and checked by methods well-known to the skilled person.

Both parts are cultured in a suitable culture medium. A suitable culture medium may be based on Dulbecco's alpha Minimal Essential Medium (α-MEM), or any other conventional suitable medium. Preferably, the medium contains additional amounts of L-ascorbic acid 2-phosphate, an antibiotic, serum, and/or a growth factor. The antibiotic is preferably chosen from the group of penicillin G, gentamicin, fungizone, and streptomycin. The growth factor is preferably basic fibroblast growth factor (bFGF). A highly preferred culture medium is a medium as described in the international patent application PCT/NL00/00958.

Since it is an important aspect of the invention that an effect of an osteogenic differentiation factor on the expression of alkaline phosphatase by the bone cell population is determined, one of the two parts of the sample of the bone cell population is cultured in the presence of an osteogenic differentiation factor, while the other is cultured without said factor. A preferred osteogenic differentiation factor is dexamethasone (dex). Dexamethasone is preferably used in an amount ranging from 10^{-10} to 10^{-5} M.

The cells are preferably cultured in from 2 to 6 passages. The number of passages can suitably be chosen by the skilled person based on his experience to obtain a large enough number of cells. During each passage, the culturing is preferably continued until a state of confluency is reached.

During culturing the cells will or will not express a certain amount of alkaline phosphatase (ALP). In accordance with the invention, this amount is determined. To this end, after culturing in sufficient passages, the cells of both parts are labelled with an antibody specific for alkaline phosphatase. A preferred example of a suitable antibody is anti-ALP (hybridoma B4-78).

The labelling of the cells with the antibody may be carried out in any conventional manner. A suitable manner to determine the degree of expression of ALP of both parts of the sample of the bone cell population is by

flow cytometry. The expression of ALP is measured as the percentage of ALP positive cells as compared to the total cell population.

In accordance with the invention it has been found that the relationship between the degrees of expression of ALP of both parts provides a prediction of the capacity of the bone. Statistical analysis has indicated that this capacity may be expressed in the form of the logarithm of the ratio of the degrees of expression of ALP of the first part, cultured in the presence of osteogenic differentiation factor, and the second part, cultured without osteogenic differentiation factor. Thus, a quantitative measure is obtained for the osteogenic potential of a cell population.

In order to determine whether the cell population is suitable to use in tissue engineering a medical implant, such as a bone construct, the obtained value may be compared to a discriminating index. The index can be obtained from statistical analysis from a significant number of experiments and has been found to lie between 0.17 and 0.23. If the value calculated for the logarithmof the ratio of the degrees of expression of ALP of the first and second parts is larger than the index, it may be assumed that the osteogenic potential of the cell population is sufficient to use said population in tissue engineering.

The invention further relates to a kit for carrying out the above described method. Said kit comprises means to provide a sample of a bone cell population, means for culturing bone cells, an osteogenic differentiation factor, an antibody specific for alkaline phosphatase. The means to provide a sample of a bone cell population preferably comprise means to take a biopsy, and the means for culturing bone cells preferably comprise a suitable culture medium.

The invention will now be further elucidated by the following, non-restrictive examples.

EXAMPLES

MATERIALS AND METHODS

Human bone marrow stromal cell (HBMSC) harvest and culture

Bone marrow aspirates (10 - 30 ml) were obtained from 14 patients that had given written informed consent. Donor information is summarized in Table 1.

Table 1: HBMSC donor information

Donor	Source of bone marrow	Gender	Age	Identification number
1	Iliac crest	M	75	U00079
2	Acetabular fossa	M	86	U00084
3	Iliac crest	M	74	U00169
4	Iliac crest	M	45	U00182-2
5	Iliac crest	F	39	U00212
6	Acetabular fossa	F	54	U00230
7	Spine	M	44	U00106
8	Iliac crest	F	69	U00173
9	Iliac crest	M	74	U00174
10	Acetabular fossa	F	72	U00178
11	Iliac crest	F	70	U00179
12	Iliac crest	F	74	U00090
13	Acetabular fossa	F	67	U00180
14	Spine	M	44	U00091

F = female M = male

The bone marrow specimens were collected in heparinized tubes and transported at room temperature. Cells were re-suspended with a 20G needle, plated at a density of 500,000 nucleated cells/cm^2 and cultured in minimum essential medium (α - MEM, Life Technologies, The Netherlands) containing 10% foetal bovine serum (FBS, Life Technologies, The Netherlands), antibiotics (AB), 0.2mM L-ascorbic acid 2-phosphate (AsAP, Life Technologies, The Netherlands) and 1ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37°C and in a humid atmosphere with 5% CO_2. The culture medium was refreshed twice a week and, at near confluence, the adherent cells were washed with phosphate buffered saline solution (PBS, Life Technologies, The Netherlands) and

enzymatically released by means of a 0.25% trypsin – EDTA solution (Sigma, The Netherlands). Cells were plated at a density of 5,000 cells per cm^2 and subsequent passages were performed when cells were near confluence (80-90%).

Scaffold material

Porous granules of coraline hydroxyapatite (HA) with an average surface area of 0.2 – 0.3 cm^2 were used as scaffold material. The interconnected pores had a median diameter of 435 μm and the size of the particles was approximately 3 x 2 x 2 mm.

Antibodies

The purified anti-ALP (hybridoma B4-78), anti-PCI (M-38) and anti-OP (MPIIIB10) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, USA). The control mouse immunoglubin G (IgG2a) monoclonal antibody and the secondary antibody goat anti-mouse IgG γ-chain-specific-FITC were purchased from Dako (Denmark).

Temporal expression of ALP

Fourth passage HBMSC (donors 1 to 14) were plated at a density of 5,000 cells per cm^2 and cultured for 8 to 9 days both) in two different types of media: (i) α - MEM containing 10% FBS, AB, 0.2mM AsAP and 0.01M β-glycerophosphate (βGP, Sigma, The Netherlands) (control medium) and (ii) control medium with the addition of 10^{-8} M dexamethasone (dex, Sigma, The Netherlands) (+ dex medium). The expression of ALP was evaluated by flow cytometry at several culture periods (from day 1 to day 9, three to four measurements were performed for each culture). Briefly, after trypsinisation, cells were washed twice in wash buffer and blocked against non-specific binding (see above). Cells (approx. 0.1-0.3E6 / staining) were then resuspended in blocking buffer containing: (a) control mouse anti-human IgG2a (1:5

dilution) and (b) ALP monoclonal antibody (1:10 dilution). After incubation on ice for 45 minutes and washing, antibody reactivity was detected by suspending the cells with blocking buffer containing goat anti-mouse IgG γ-chain-specific-FITC (1:5 dilution). Cells were incubated on ice and in the dark for 30 minutes. After washing, the cells were resuspended in 200μl of FACS-flow/staining and analyzed using a FACS Calibur apparatus (Becton Dickinson Immunocytometry systems). For each measurement 10,000 events were collected..

In vivo osteogenic potential of HBMSC

HBMSC (passage 4, donor 1 to 14) were seeded on porous HA granules, at a density of 200,000 cells/particle and cultured for one week in (+) dex medium. Following this period, and prior to implantation, the tissue engineered samples were soaked in serum free medium and washed in phosphate buffered solution pre-warmed to 37°C. Samples (n = 6 per donor) were then implanted into subcutaneous pockets created in the back of immunodeficient mice. Samples of each culture were divided over two animals. At the end of the six-week survival period, the implants were removed and fixed in 1.5% glutaraldehyde in 0.14 M cacodylic acid buffer, pH 7.3. The fixed samples were dehydrated and embedded in methyl methacrylate. The sections were processed undecalcified on a histological diamond saw (Leiden microtome cutting system) and then stained with basic fuchsin and methylene blue in order to detect bone formation.

Statistics

Statistical analysis was performed using both t student tests and Mann-Whitney U tests assuming non equal variances. Statistical significance was defined as $p<0.05$. When calculating an index to predict osteogenic capacity, an asymptotic curve test was also performed to analyse statistical significance between the ROC curve and the curve of no discrimination.

RESULTS

Temporal expression of ALP

In HBMSC cultures from each donor, the temporal evolution pattern of ALP+ cells was similar in both culture conditions. However, in cultures treated with dex, the fraction of ALP+ cells was consistently higher as compared to control cultures (fig. 1a). Statistical analysis revealed that after the first two days of culture, the proportion of ALP+ cells in the (+) dex condition was significantly higher as compared to the control ($p<0.05$), revealing that dex stimulation induced an increase in the fraction of committed osteoprogenitor cells. In the majority of the donors tested (12 of 14), the relative amount of ALP+ cells increased during culture period reaching a maximum value and decreased thereafter. The time period required to achieve the maximum of ALP expression, as well as the value of the maximal fraction of ALP+ cells, was affected by the culture conditions and markedly donor dependent (fig. 1a and b). In HBMSC cultures from 2 of the 14 patients, the percentage of cells expressing ALP was above 80% in the beginning of the culture and decrease thereafter (data not shown).

Quantification of osteoprogenitor cells in culture

An approach that may allow for the indirect quantification of osteoprogenitor cells is the degree of culture stimulation by dex with regard to the fraction of ALP+ cells. That is, cultures exhibiting a high fold increase in the amount of cells expressing ALP due to dex treatment most likely contain a higher proportion of osteoprogenitor cells as compared to cultures in which stimulation by dex induces a lower fold increase in ALP expression. Therefore, for each donor and culture period, the degree of stimulation by dex was measured through the ratio between the fraction of ALP+ cells in the (+) dex and control conditions. Both t student and Mann-Whitney U tests indicated

that, after the first two days in culture, this ratio was time independent, révealing that the optimal cell response to dex treatment occurred after the first 48 hours. To verify whether the degree of culture response to dex was correlated to the *in vivo* bone induction ability of the cultures, for each donor the average ratio was determined using the measurements performed from day 3 to day 9 (Table 2). This ratio, taken as an indirect measure for the proportion of osteoprogenitor cells, was then compared to the *in vivo* osteogenic potential of the cultures.

Table 2 – Degree of dex stimulation measured as the ratio between the fraction of ALP^+ cells in the (+) dex and control conditions.

Donor	Ratio	Identification number
1	1.53	U00079
2	1.53	U00084
3	1.40	U00169
4	2.71	U00182-2
5	2.53	U00212
6	1.52	U00230
7	1.25	U00106
8	3.72	U00173
9	1.56	U00174
10	2.12	U00178
11	2.40	U00179
12	2.56	U00090
13	1.55	U00180
14	1.80	U00091

In vivo osteogenic potential of HBMSC

To determine the *in vivo* bone inducing ability of HBMSC cultures, cells were seeded into porous HA, at a density of 200,000 cells/ particle and further cultured for one week, in the presence of dex. Following this period, the samples were implanted under the skin of immunodeficient mice. Six weeks post implantation, *de novo* formed bone was found in all the samples from 8 of the 14 assessed donors (1-2, 4-5, 8, 11-12, 14). Woven, mineralized bone tissue was observed in direct contact with the ceramic material, indicating that the implanted cells survived and further differentiated into osteoblasts. The bone

matrix displayed embedded osteocytes and blood vessels were often observed close to the newly deposited bone. The HBMSC cultures from these donors revealed a good agreement between the *in vivo* and vitro data, in which the osteogenic character of the cultures was demonstrated by the expression of PCI, OP (donor 1, 2, 4, 5) and by an increase in ALP expression after treatment with dex. However, and although *in vitro* testing also indicated expression of PCI, OP (donor 3, 6, 7) and an increase in ALP expression after treatment with dex, HBMSC cultures from donors 3, 6, 7, 9, 10 and 13 failed to induce *in vivo* osteogenesis.

In vivo osteogenic potential versus degree of stimulation by dex with regard to ALP expression

The *in vivo* bone formation capacity of HBMSC could not be related to their *in vitro* expression of PCI, OP or ALP. However, the relative increase in the proportion of ALP+ cells in culture following dex treatment proved to be related to the *in vivo* bone induction capacity of the cultures, revealing that these relative increase, expressed by the ratio between the fraction of ALP+ cells in (+) dex and control conditions, can be taken as an indirect measurement for the proportion of osteoprogenitor cells in culture. Our data demonstrated that the degree of dex stimulation was higher in bone forming cultures as compared to cultures that failed to induce osteogenesis (fig. 2). Both t student and Mann-Whitney U tests revealed a statistically significant difference between bone forming and non bone forming cultures with regard to the increase on ALP expression after dex treatment ($p = 0.021$, t student test; $p = 0.029$, Mann-Whitney U test).

Following these results, we performed a preliminary attempt to define an index to predict *in vitro* the *in vivo* performance of the implant. Statistical analysis indicated that this index should be based on the log of the ratio between the proportion of cells expressing ALP in the (+) dex and control condition. This parameter displayed the smallest variance and the best

discrimination in the t test ($p = 0.016$ for log ratio and $p = 0.021$ for ratio). Therefore, these values were calculated for each donor and the best discriminating index was determined using the so called ROC curve (fig. 3). The test result for the log ratio revealed a statistical significance ($p = 0.028$, asymptotic curve test) between the obtained ROC curve and the curve of no discrimination (fig. 3). The data further revealed that the best index should be 0.20, meaning that in order to obtain *in vivo* bone induction by HBMSC log ratio should be equal or higher than 0.20. This index, and for the donor population assessed, provided a correct prediction (sensitivity) in 75% of the cases and an accuracy in classifying non bone forming cultures (specificity) of 83.3%.

LEGENDS TO FIGURES

Figure 1 –Temporal expression of ALP in HBMSC cultures: Effect of dexamethasone treatment and variance between donors. (a) Donor 11 and (b) Donor 9.

Figure 2 –Relative increase in the fraction of ALP^{+} cells in bone forming and non bone forming cultures, after dex treatment. (◊) Individual values of 14 donors; (♦) Average of each population; (*) Statistical significance was observed: $p = 0.021$ in t test and $p = 0.029$ in Mann-Whitney U test.

Figure 3 – Determination of the osteoinductive index by a ROC curve analysis. Sensitivity was defined as the ratio between correctly predicted cases and the total number of cases. Specificity was defined as the ratio between correctly predicted non bone forming cases and the total number of cases.

Claims

1. A method for determining *in vitro* the capacity of a cell population to induce bone formation *in vivo* comprising the steps of:

a) providing a sample of a cell population;

b) dividing said sample into a first and a second part containing an equal number of cells;

c) culturing the first part in the presence of an osteogenic differentiation factor;

d) culturing the second part in the absence of an osteogenic differentiation factor;

e) labelling the cells of the first and second parts with an antibody specific for alkaline phosphatase;

f) quantitatively determining degrees of expression of alkaline phosphatase expressed by the cells in each part; and

g) comparing the degrees of expression of alkaline phosphatase of the first part and the second part thereby providing a measure for the capacity of the bone cell population to induce bone formation *in vivo*.

2. A method according to claim 1, wherein the sample of a cell population is obtained through a biopsy from a patient who has to undergo surgery to receive a bone implant.

3. A method according to claim 2, wherein the cell population comprises human bone marrow stromal cells, and/or human osteoprogenitor cells.

4. A method according to any of the preceding claims, wherein the osteogenic differentiation factor is dexamethasone.

5. A method according to claim 4, wherein the dexamethasone is used in an amount of 10^{-10} to 10^{-8} M.

6. A method according to any of the preceding claims, wherein the cells are cultured in from 2 to 6 passages.

7. A method according to any of the preceding claims, wherein the cells are cultured in a culture medium based on α-MEM.

8. A method according to claim 7, wherein the culture medium further comprises L-ascorbic acid 2-phosphate, an antibiotic, serum, and/or a growth factor.

9. A method according to claim 8, wherein the growth factor is basic fibroblast growth factor (bFGF).

10. A method according to claim 8, wherein the antibiotic is chosen from the group of penicillin G, gentamicin, fungizone, and streptomycin.

11. A method according to any of the preceding claims, wherein the antibody is anti-ALP (hybridoma B4-78).

12. A method according to any of the preceding claims, wherein the degrees of expression of alkaline phosphatase are determined by flow cytometry.

13. A method according to any of the preceding claims, wherein step g) comprises calculating the logarithm of the ratio of the degrees of expression of alkaline phosphatase of the first part and the second part.

14. A method according to claim 13, wherein said logarithm is compared to a best discriminating index.

15. A method according to claim 14, wherein the best discriminating index has a value between 0.17 and 0.23.

16. A kit for carrying out a method according to any of the preceding claims, comprising means to provide a sample of a bone cell population, means for culturing bone cells, an osteogenic differentiation factor, an antibody specific for alkaline phosphatase.

17. A kit according to claim 16, wherein the means to provide a sample of a bone cell population comprise means to take a biopsy.

18. A kit according to claim 16 or 17, wherein the means for culturing bone cells comprise a culture medium.

EPO - DG 1

08. 06. 2001

(75)

Title: Assay for predicting cell activity

Abstract

The invention relates to a method for determining *in vitro* the capacity of a cell population to induce bone formation *in vivo* comprising the steps of:

a) providing a sample of a cell population;

b) dividing said sample into a first and a second part containing an equal number of cells;

c) culturing the first part in the presence of an osteogenic differentiation factor;

d) culturing the second part in the absence of an osteogenic differentiation factor;

e) labelling the cells of the first and second parts with an antibody specific for alkaline phosphatase;

f) quantitatively determining degrees of expression of alkaline phosphatase expressed by the cells in each part; and

g) comparing the degrees of expression of alkaline phosphatase of the first part and the second part thereby providing a measure for the capacity of the bone cell population to induce bone formation *in vivo*.

EPO - DG 1

08. 06. 2001

(75)

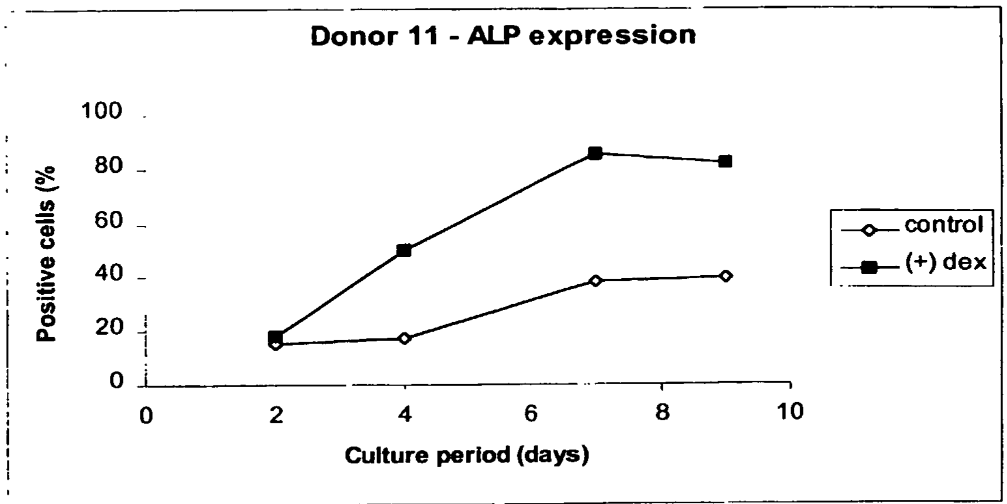


Figure 1(a)

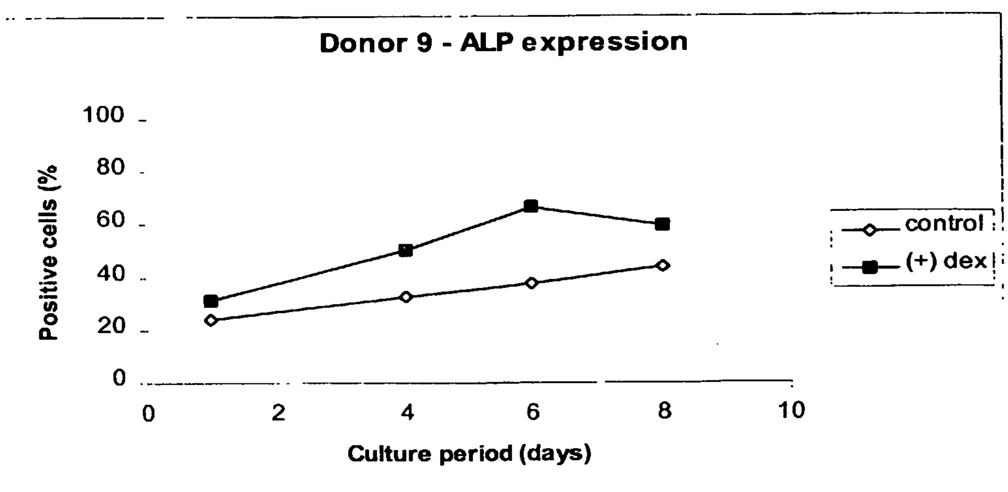


Figure 1(b)

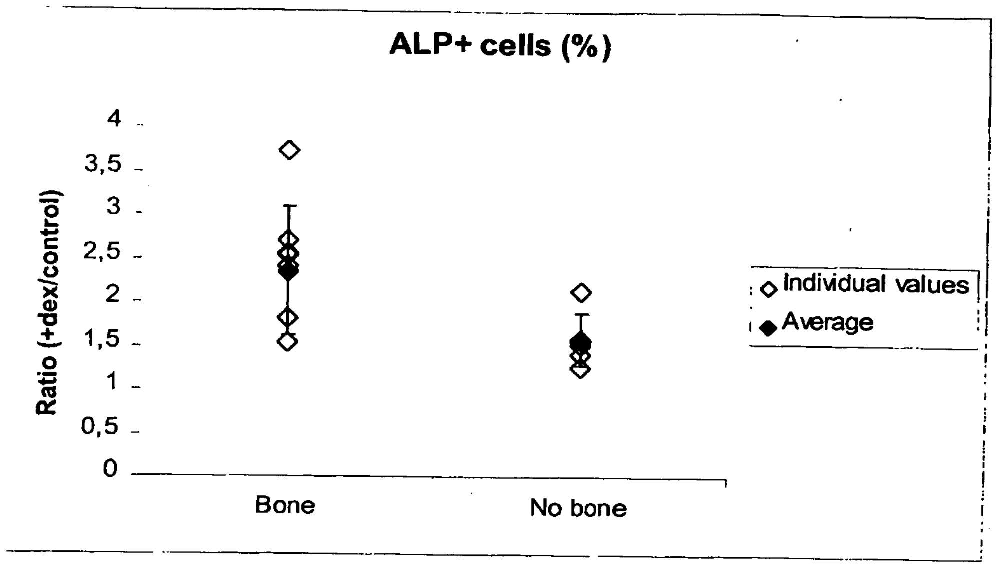
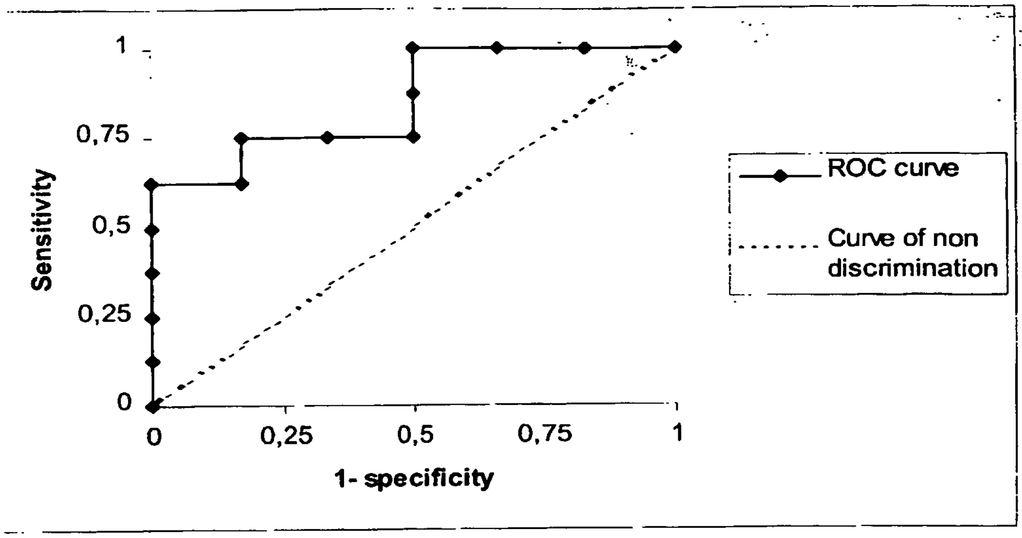

ALP+ cells (%)
Ratio (+dex/control)
4
3,5
3
2,5
2
1,5
1
0,5
0
Bone
No bone
Individual values
Average

Figure 2



1
0,75
0,5
0,25
0
Sensitivity
0
0,25
0,5
0,75
1
1- specificity
ROC curve
Curve of non discrimination

Figure 3